

TECHNIQUES FOR THE COLLECTION AND ASSAY OF
SLEEP PROMOTING FACTOR IN EXERCISED AND
SLEEP DEPRIVED DOGS

An abstract of a Thesis by
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The problem. The Pieron Phenomenon is the production of sleep in fully rested animals after a ventricular infusion of cerebrospinal fluid (CSF) from sleep deprived animals. According to the humoral theory of sleep, this is caused by the presentation of some sleep promoting substance (factor S) which depresses the brain. The somnolence produced is accompanied by an increase in slow wave sleep (SWS). Other factors which also increase SWS, such as exercise, may also do so through some mechanism involving the accumulation of factor S. Although this molecule is known to be a polypeptide with a molecular weight under 500, the structure remains unknown, greatly complicating the exploration of these relationships.

Procedure. Schedules and equipment which permit controlled administration of exercise and sleep deprivation in dogs, and techniques for the removal and storage of CSF were developed and described. An assay procedure was described which makes use of the Pieron Phenomenon and the diurnal cycle of locomotor activity in rats.

Summary. This paper describes several techniques which, when appropriately combined, can be incorporated into subsequent experiments measuring the effects of a variety of parameters on the production of sleep promoting substance in the CSF.

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SLEEP DEPRIVED DOGS

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INTRODUCTION

The "hypnotoxin" or "humoral" theory is one of three proposed sleep mechanisms. It postulates the production and accumulation of certain neuroactive substances, supposedly end product of metabolism, either in the tissues in general, or in the brain. The brain is depressed when the concentration of such substances reaches a threshold level. The gradual removal of these substances during sleep leads to the return of wakefulness. This paper deals with a class of observations related to this theory called the Pieron Phenomenon.

More than 60 years ago, Henri Pieron infused cerebrospinal fluid (CSF) from dogs deprived of sleep for relatively long periods of time into the ventricular system of normal animals. The recipient dogs showed sleep, or significantly depressed activity, for several hours following the infusion. It was proposed, in keeping with the humoral theory, that some hypnotoxic substance was produced and released into the CSF of the animal during sleep deprivation. The subsequent infusion of this "sleep deprived" CSF into the recipient animals caused changes in their behavior due to the high concentration of the hypnotoxin. However, because Pieron's techniques involved inflicting a considerable amount of stress upon the animals, his results were regarded as possible artifacts of the procedure, and the

significance of the Pieron Phenomenon was questioned.

J. R. Pappenheimer devised experiments that confirmed the essential observations described by Pieron under conditions involving much less stress to the experimental animals. His results indicate that there is a gradual release of some humoral factor, dubbed factor S by Pappenheimer, into the CSF during the first 24 hours of sleeplessness, suggesting that it may somehow regulate the normal cycle of sleep and wakefulness. Furthermore, CSF collected from goats promoted sleep in rats and rabbits, suggesting that the same, or similar, humoral factor may operate in various mammalian species. Though the factor S molecule itself has not yet been identified, the physiological mechanism for the production, release, and accumulation of this substance seems a fruitful area for research.

Sleep deprivation has been shown to cause an increase in the amount of slow wave sleep (SWS) in the recipient animals mentioned above (Pappenheimer, 1976), and in the amount of compensatory SWS in the donor animals (Berger and Oswald, 1962). According to the humoral theory, both operate through some mechanism promoting the accumulation of factor S. Other factors have also been associated with increased SWS. The normal proportion of SWS has been shown to be greatest in infancy and childhood (Roffwarg et al., 1964), progressively less from sixteen to sixty

(Williams et al., 1966), and least in old age (Kales et al., 1966). It has been proposed that SWS is related to fatigue (Roffwarg et al., 1966), to physiological repairative processes and energy build up (Fisher, 1965), and to some debt incurred during wakefulness (Oswald, 1962). Finally, work has been done suggesting a general positive relationship between exercise and the amount of SWS in a night's sleep (Baekeland and Lasky, 1966).

Since factor S accumulation, as indicated by sleep deprivation, is accompanied by increased SWS, it is possible that the other SWS increasing parameters mentioned above may also be related to the factor S mechanism. If these parameters increase SWS through some mechanism related to factor S, then the overflow of factor S into the CSF may be adequate to allow detection by infusing CSF from donor animals into recipients and quantitatively measuring the effects of the infusion.

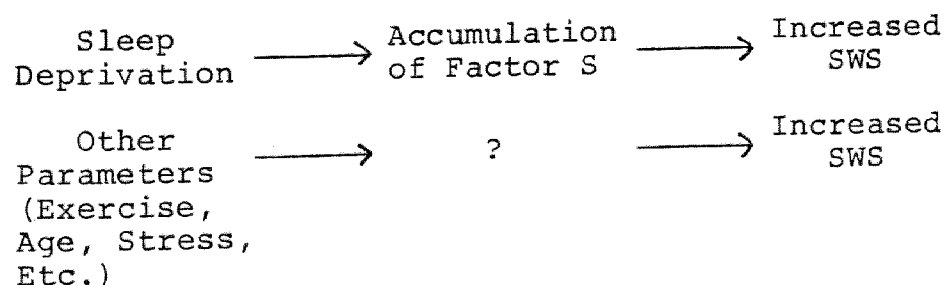


Figure 1. Possible relationship of sleep deprivation and other parameters to increased SWS, according to the humoral theory of sleep.

The purpose of this thesis is twofold. The first step is to propose an experimental design which evaluates the relative effectiveness of exercise and sleep deprivation in promoting factor S production. This is described in the experimental design section. The second step is to develop, build, and test all of the hardware and techniques which are necessary to carry out this experimental design. This is described in the Methods and Materials section and the Results section.

EXPERIMENTAL DESIGN

The project consists of two parts. The first entails manipulation of the experimental parameters of exercise and sleep deprivation to produce a characteristic concentration of factor S in the CSF of the donor animal. The second part is an evaluation of the sleep promoting tendency of a sample of this CSF, accomplished by infusing it into the ventricular system of recipient animals and measuring the resultant changes in sleep patterns. Because Pappenheimer showed that factor S is not significantly species specific in mammals, considerable freedom can be exercised in the choice of animals to be used. Dogs are used in this design as donors because of the ease of handling them and the relatively large volume of obtainable CSF. Rats are used as recipients because of their well defined diurnal cycle of sleep and activity.

The procedural path of the factor S produced in the donor animals is illustrated in Figure 2. The rate of production or accumulation of factor S in the CSF is a function of the procedure the donor dog experiences. Before either the brain can metabolize the factor S or the concentration is diluted by normal CSF flow, a 4 ml sample is removed from the cisterna magna and stored at -20°C until utilized in the assay procedure. Quantitative evaluation of the effect is accomplished by infusing the sample into the ventricular system of the recipient animals and measuring the changes produced in their locomotor activity during the subsequent 24 hour period. This data is compared with control values for that same rat. In accordance with the Pieron Phenomenon, CSF from the dog infused into the rat should cause an increase in the amount of SWS in the rat and, therefore, a measurable decrease in the amount of locomotor activity from normal. The more factor S that is present in the donor CSF, the greater the depression of the rat's activity.

Acclimation Period. It is essential to stabilize the sleep-wakefulness cycle of the donors so that the onset of wakefulness and sleep can be closely estimated. The animals are placed on a 12-12 hour light-dark cycle, the light phase beginning at 0600 and the dark phase at 1800. This cycle is maintained throughout the experiment with food and water supplied ad lib. The onset of wakefulness, or

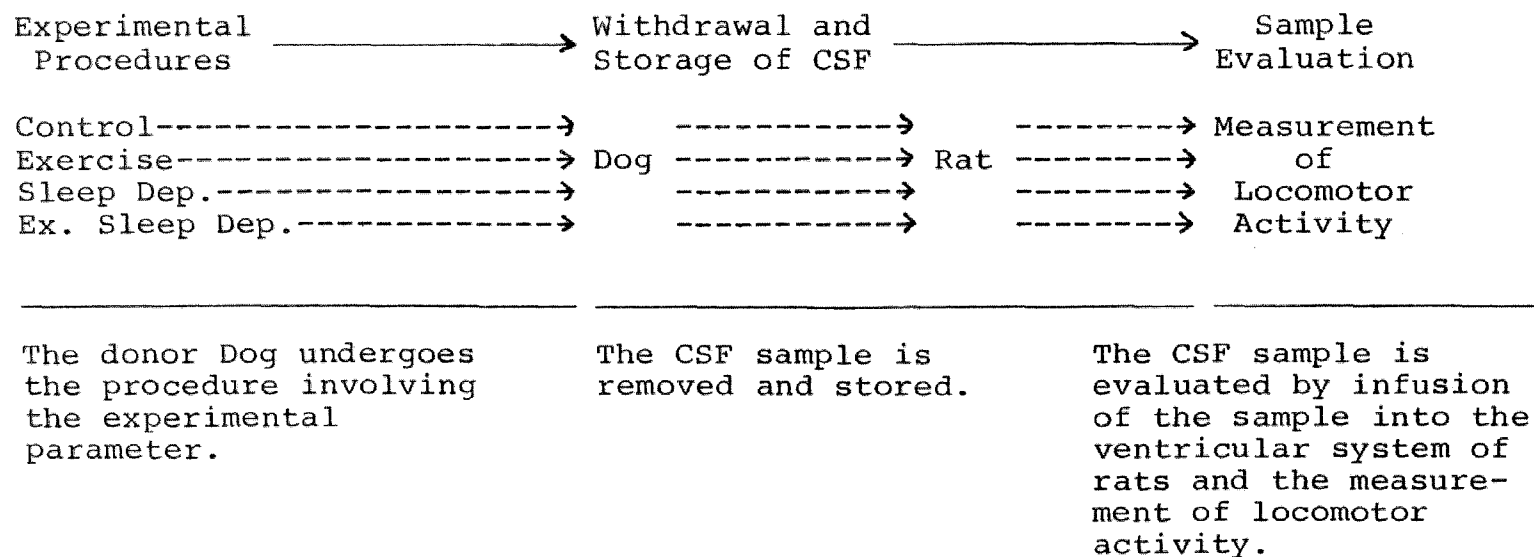


Figure 2. The procedural path and treatment of CSF through donor and recipient animals.

the time from which the number of hours since last sleep will be calculated, is designated as the beginning of the light phase. The dogs are acclimated to this cycle for a two week period to allow adjustment of their diurnal cycles.

Manipulation and Comparison of Experimental

Parameters. The following guidelines are used in designing experimental procedures. (1) All procedures begin at 0600. This assumes that when the lights come on at 0600, the dog awakens from a complete night's sleep and has a minimal concentration of factor S in its CSF. (2) To eliminate variation between donor CSF samples, each dog supplies a control CSF sample as well as one for each experimental procedure, and the data obtained is compared only with data from that same dog. (3) The order in which each dog experiences these procedures is randomized to eliminate the possible influence of order or learning on the data. (4) Procedures which are to be compared must be identical except for the variation of one experimental parameter. (5) A dog which has experienced a dural puncture does not undergo another puncture for a two week period to prevent the development of adhesions and to insure full recovery.

A control and three experimental procedures designed to explore the relationship of exercise and sleep deprivation to the factor S mechanism are described below. Figure 3 further illustrates these procedures.

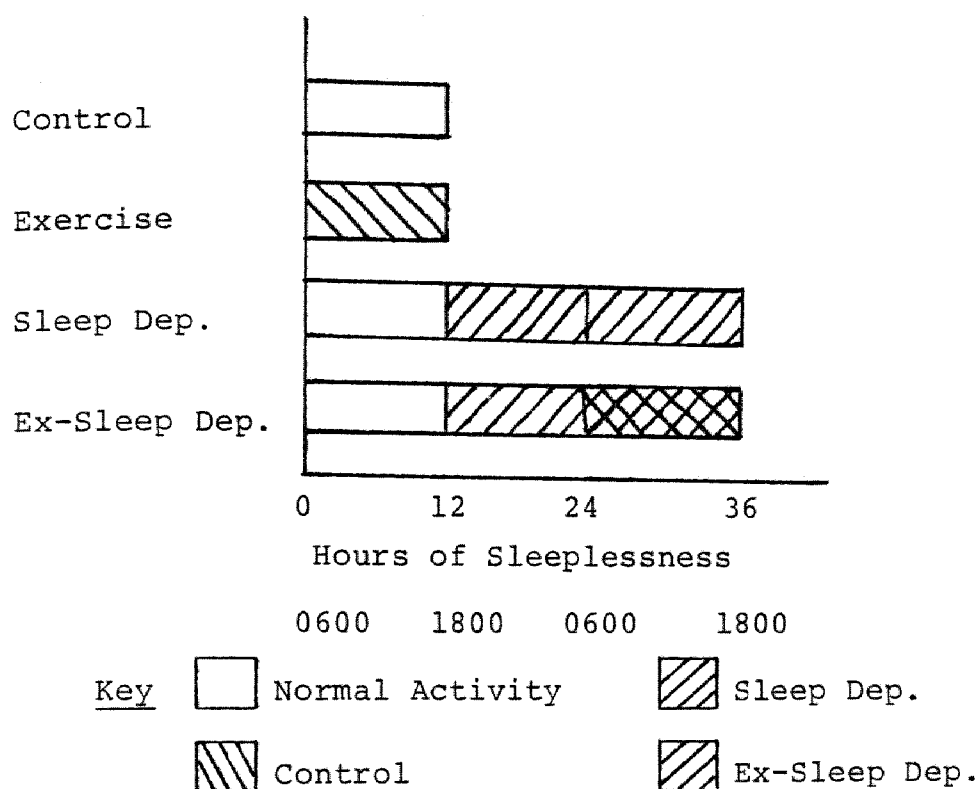


Figure 3. Amounts, types, and times of stimuli for experimental procedures.

Control. At 0600, the lights in the animal room are turned on, awakening the dog. The animal is confined to its cage for the duration of the light phase with food and water supplied ad lib. At 1800, 4 ml of CSF is withdrawn from the cisterna magna and frozen at -20°C .

Exercise. The dog is awakened at 0600 and immediately placed on the treadmill for exercise. Each 60 minute exercise session is followed by a 60 minute rest period, during which the dog is returned to its cage. This exercise-rest cycle continues throughout the light phase, accumulating a total of six hours of exercise. At 1800,

4 ml of CSF is removed and frozen.

Sleep Deprivation. The dog is awakened at 0600 and confined to its cage until 1800 as in the control procedure. The dog is then deprived of sleep during the following 24 hours. At 1800 of the following day, 36 hours after the donor last slept, 4 ml of CSF is removed and frozen.

Exercise and Sleep Deprivation. The dog awakens at 0600 and until 1800 is confined to its cage as in the control procedure. From 1800 to 0600 of the following day, the normal dark phase, the dog is deprived of sleep. At 0600, the animal is placed on the treadmill and undergoes the exercise-rest cycle described for the exercise procedure. At 1800 of the second day, 36 hours after the dog last slept, 4 ml of CSF is removed and frozen.

This project is designed to compare the sleep promoting effectiveness of CSF obtained from each of the experimental procedures on recipient rats. Table 1 presents the amount of time devoted to each parameter during the above procedures. The effect of exercise on the production of factor S is observed by comparing the exercise and control procedures. Data from the sleep deprivation procedure is compared with the control data to confirm the basic observations of the Pieron Phenomenon. Comparison of the exercise and sleep deprivation data with the control will provide information about the relationship of these stimuli to the factor S mechanism, and about the potency

of exercise, as compared to sleep deprivation, in promoting sleep. Finally, a comparison of the data from the sleep deprived and exercise-sleep deprived groups with the control explore the possible cumulative effect of exercise and sleep deprivation in promoting sleep.

Table 1. Comparative amounts of exercise and sleep deprivation for the experimental procedures.

Experimental Procedure	Amount of Exercise (hrs)	Amount of Sleep Dep. (hrs)
Control	0	12
Exercise	6	12
Sleep Dep.	0	36
Ex-Sleep Dep.	6	36

Assay of Sleep Promoting Factor in CSF. A fairly simple assay of the sleep promoting characteristics of a CSF sample was devised by Pappenheimer based upon the amount of locomotor activity rats exhibit in the light and dark phases of their diurnal cycles. Rats sleep about 65% of the time during the day and 30% of the time at night. The assay involves the infusion of the suspected sleep factor two hours before the beginning of the dark phase. The exogenous or non-native sleep inducing substance overcomes the animal's natural wakefulness, causing it to sleep more and, hence, be less active at night.

As shown by Pappenheimer with simultaneous recordings, the reduction of locomotor activity following an infusion of CSF from sleep deprived animals is associated with an increase in the duration of SWS measured on the basis of continuous EEG recordings. This assay of locomotor activity is useful in screening large numbers of samples where EEG measurement is inconvenient or impractical.

A slightly modified form of Pappenheimer's technique is used in this project. The rats are acclimated to a 12-12 light-dark cycle similar to that of the dogs. Each rat is implanted with a ventricular guide tube and allowed to recover before control locomotor activity data is collected. The CSF sample is infused into the lateral ventricle of the rat just prior to the dark phase of its diurnal cycle. The rat is returned to its cage, and locomotor activity is measured for the following 24 hours. The data collected is compared with control data from that same rat and analyzed for significance.

METHODS AND MATERIALS

Factor S Donors

Delivery of Experimental Parameters. The apparatus used to deliver each experimental parameter must not only be durable and able to perform effectively, but also must

insure uniformity of delivery between animals and eliminate novel stimuli.

Sleep Deprivation Device. A device similar to that used by Pappenheimer with goats, which deprives dogs of sleep by forcing them to remain in a standing position, was devised by this researcher. If the animal lies down, a mild shock is delivered to its tail through attached electrodes. When the dog rises, the stimulation ceases.

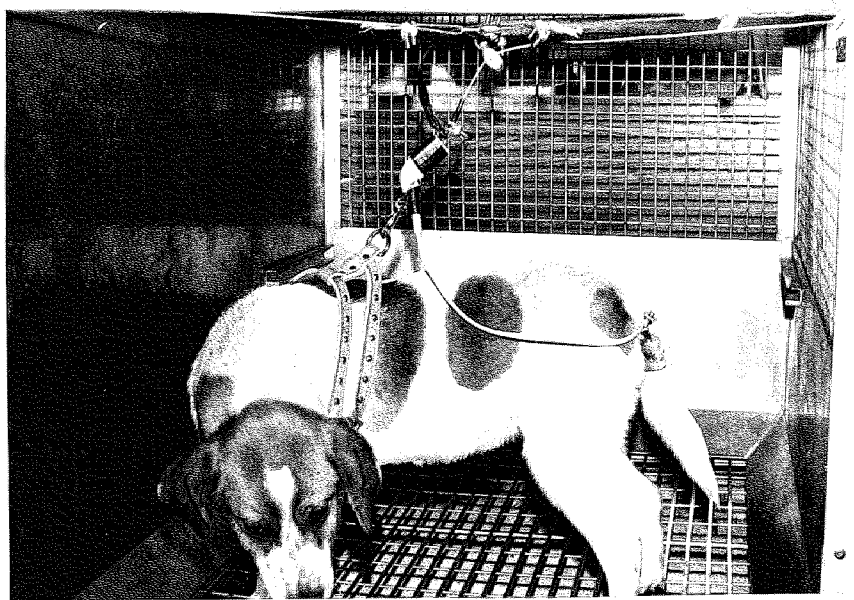


Figure 4. A dog wearing the harness and shock plates of the sleep deprivation device.

The construction and operation of this device is illustrated in Figure 5. A cable system rigged across the ceiling of the cage suspends a pulley from the center; through this a cable passes from the dog's harness to a mercury switch situated outside of the cage. When the dog lies down, the cable is pulled and the mercury switch is

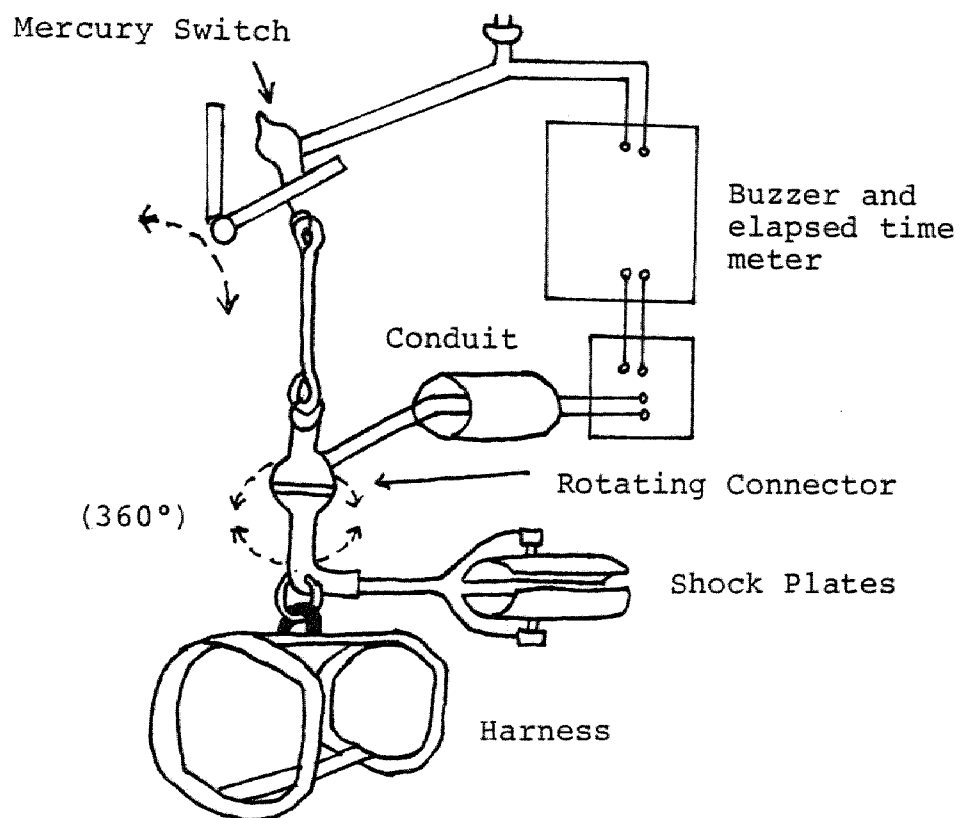


Figure 5. Construction and operation of the sleep deprivation device.

closed. It is returned to an open position by a spring as the animal rises. The switch completes a circuit containing a buzzer, an elapsed time meter, and a stimulator. When the switch is closed, the buzzer sounds and a shock is delivered through electrodes attached to the animal's tail. The elapsed time meter measures the amount of time that current is delivered to the animal.

A connector permitting 360° rotation of the tail plate wires around the main cable eliminates tangling and displacement of the tail plates. All wires are passed to the outside through conduit to prevent the dog from chewing through them. The shock plates, prepared from common laboratory EKG plates, are coated on the edges with a silicone sealer to prevent current flow in the event they come into contact with each other. They are attached to the dog's tail after the area has been shaved; EKG paste is applied to promote conduction through the skin. The plates are to be secure but not interfere with the circulation of the tail. (See Figure 6.)

Exercise Apparatus. A treadmill is used to deliver uniform amounts of exercise to the dogs. It is constructed to run the animal at five mph, and is equipped with a rheostatic switch which allows the floor speed to be slowly increased from zero to five mph. The running surface measures 110 cm by 48 cm. The treadmill is equipped with a stall measuring 92 cm by 51 cm by 69 cm with doors on the

front and back. The ceiling consists of bars which enable the trainer to work with the dog while the treadmill is running. (See Figure 7.)



Figure 6. Proper placement of shock plates on dog's tail.

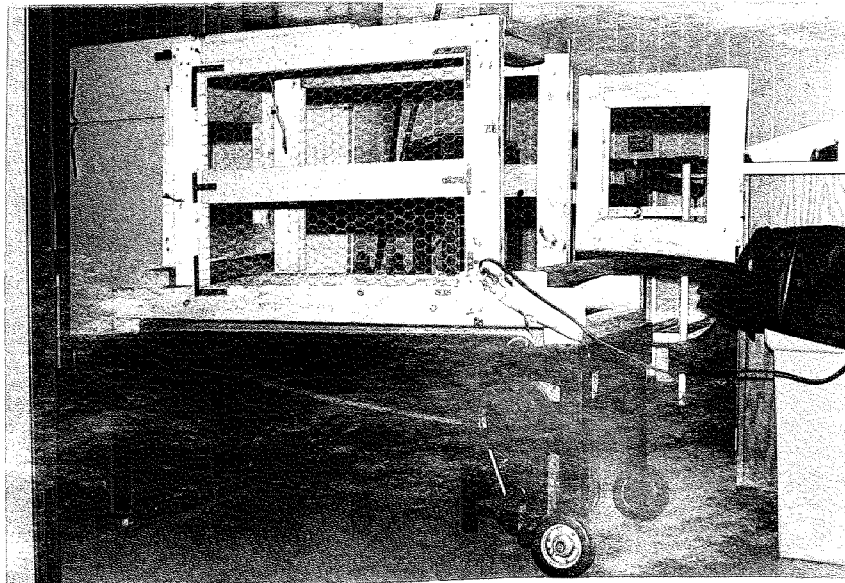


Figure 7. Treadmill of the design and dimensions described for this project.

Figure 8 illustrates the safety cable which passes from the front of the cage to the dog's harness. This supports the animal should problems arise and he be unable to keep up with the treadmill.

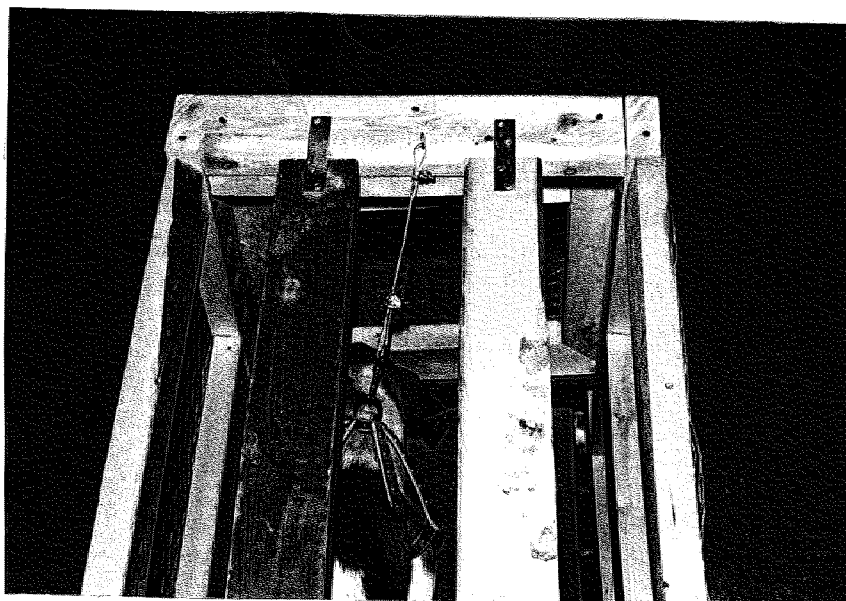


Figure 8. The safety cable, passing from the front of the cage to the harness.

The dogs are exercised for 30 minutes three times a week throughout the experiment to maintain physical condition.

CSF Withdrawal and Storage. The procedure used to remove CSF from the donor animals must meet several criteria. (1) To minimize any influence the withdrawal procedure might have upon the factor S mechanism, the process must be brief. (2) Because the donor animals are used for several cisternal punctures, it must be relatively safe.

(3) For convenience, it must require a minimum of preparation for the animal. (4) It can use no drugs that cross the blood-brain barrier before the sample has been withdrawn. Such drugs could pass into the CSF and alter the effect of the hypnotoxin infused into the rats.

The techniques used in previous projects to withdraw CSF from the donor animals are unsatisfactory for use in this project. The technique used by Pieron entailed puncturing the atlanto-occipital membrane at the base of the brain without anesthetics: the animals struggled violently and often collapsed or were partially paralyzed after the puncture. Because of the injury risk factor involved with this technique, it cannot be used in its present form.

Pappenheimer devised techniques for perfusing the cavities of the brain in specially prepared unanesthetized goats. This technique allows the membranes over the cisternal cavity to be punctured at will through a chronically implanted guide tube. CSF can then be withdrawn continuously without disturbing the animal. The horns of the goat provide mechanical protection for the guide tube and serve as an anchoring site for the infusion and withdrawal apparatus. Since this project utilizes dogs as donor animals, the anchoring site is absent negating the use of this technique.

In this laboratory, a technique has been devised using the basic aspects of Pieron's procedure. The major problem with his technique, the animal struggling during the CSF withdrawal, is overcome by the administration of curare. Curare is not an anesthetic and is not used as such here. Curare paralyzes the dog by blocking the neural synapse, and does not cross the blood-brain barrier. Its effects are reversible with other drugs. It is used to avoid the excessive struggling the animal is likely to make to the novel experience of the cisternal puncture. This technique fulfills the aforementioned criteria for the withdrawal procedure and affords several advantages over those previously mentioned. First, curare is fast acting. It paralyzes the animal within three minutes of its administration when given in the proper dosage. Second, it is progressive in action affecting different muscle groups at different rates. The ocular and facial muscles are the first affected, followed by the head and neck muscles, the limb muscles, and finally the respiratory muscles five to seven minutes after the first signs of paralysis. Third, the effects of curare are reversible with an antidote. Neostigmine rapidly reverses the effects of the curare when administered in the proper dosage with few side effects. Finally, curare does not crowd the blood-brain barrier. This insures that the drug has no effect upon the data.

The disadvantage of this technique is that because curare does not affect the consciousness of the animal, the dog is awake, though immobile, throughout the withdrawal process. The discomfort of the cisternal puncture is a form of stress. Because it occurs so near the time of sample withdrawal, however, its influence upon the CSF sample is presumed inconsequential.

A list of the materials needed for this procedure is provided in Appendix 1.

The dog is administered 0.15mg/kg of tubocurare intravenously and observed for the expected effects. If needed, a second 0.15mg/kg dose may be given in the same manner.

When the dog is immobilized, the notch between the atlas and axis is palpated and a spot one cm from the midline is marked. A 20 gauge needle with a sterile 5 ml syringe is inserted at this point until a "pop" is felt as the needle passes through the dura mater. To test if the probe is in cisterna, the plunger of the syringe is withdrawn. If the puncture is successful, CSF will pass into the syringe. If the puncture is unsuccessful, the syringe will be difficult to draw and no CSF will pass into the syringe barrel. In this situation, the needle tip is passed either deeper or shallower and tested again. Once CSF is obtained, a 4 ml volume is withdrawn and the needle removed.

Immediately after the needle is removed from the cisterna magna, 0.5 ml of 0.1% Neostigmine Methyl Sulfate is administered to the dog intraveinously. This reverses the paralyzing effects of the curare. The total time from the curare injection to the administration of the Neostigmine must not exceed five minutes, after which the effect of the curare upon the respiratory muscles is more pronounced and the chances of permanent injury to the dog resulting from anoxia are greatly increased. After normal motor control returns, the dog is returned to its cage. The CSF sample is transferred to a sterile vial, labelled, and frozen at -20°C .

Factor S Recipients

Activity Cages. The activity cages used in this project to measure the locomotor activity of rats are a modified version of those used by Pappenheimer. Each cage, illustrated in Figures 9 and 10, is criss-crossed by two beams of dim red light focused on photoconductive cells. When a beam is broken, its photocell triggers a relay which deflects the pen on a strip chart recorder. When the beam is restored, the pen returns to its original position. To conserve paper, yet adequately separate the pen deflections, the paper speed is set at 1 mm/sec. The record is analyzed for the number of times the beam is broken and restored per hour time period. This information is

separated into six hour increments starting at 1800.

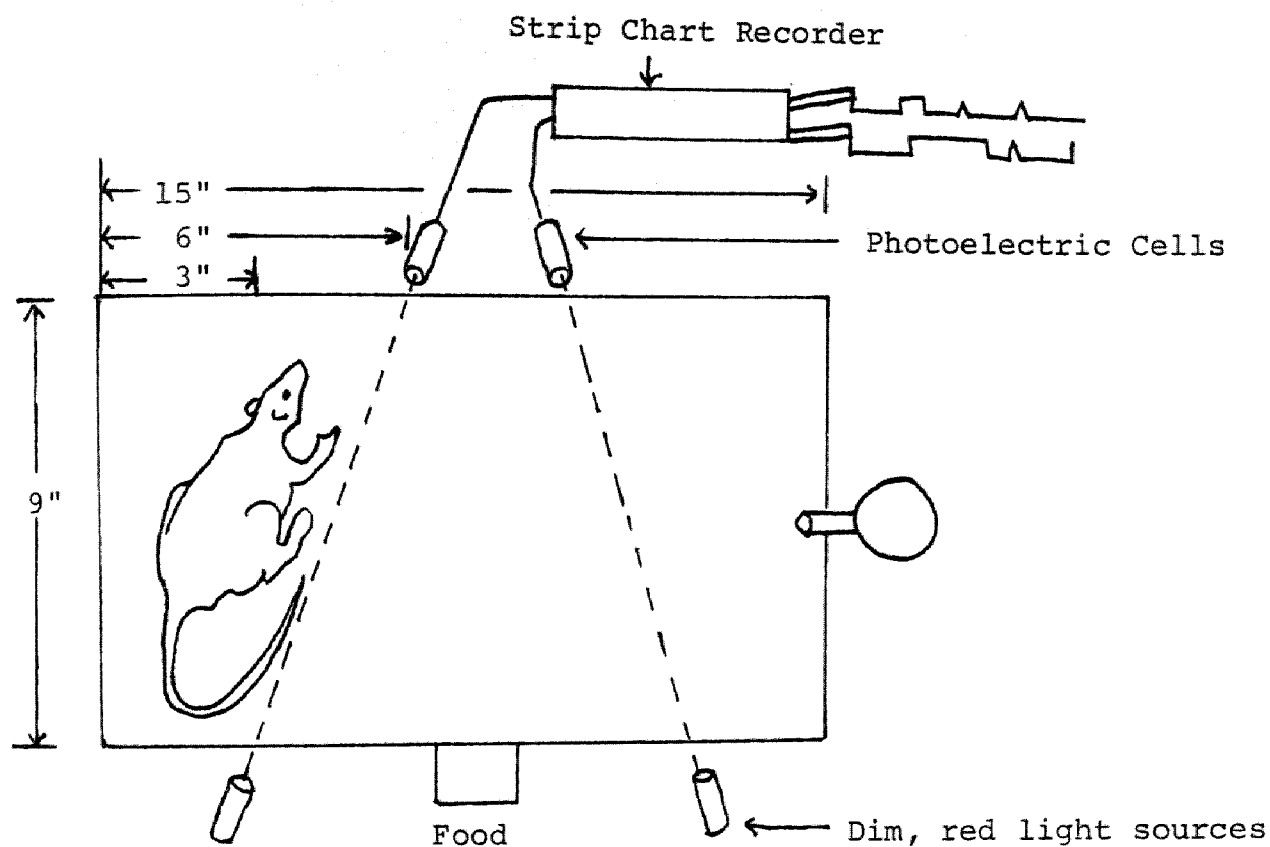


Figure 9. Design and dimensions of the locomotor activity cage.

Construction of the Guide Tube-Infusion System.

Pappenheimer used separate techniques for the implantation of the ventricular guide tubes in rats and the measurement of the length of ventricular probe needed for each rat. A technique was devised which incorporates both of these procedures into one.

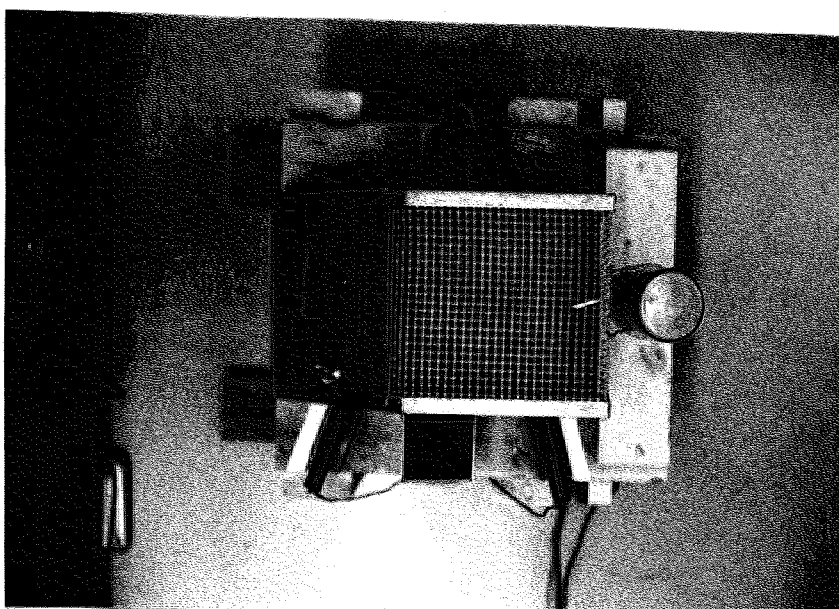


Figure 10. Top view of activity cage.

Guide Tube. The guide tube is built from a #440 brass bolt. The bolt is cut into 6 mm lengths and the center of each is drilled out to a diameter of $1/16$ on an inch. Care is taken to insure that all threads, especially the starter threads at either end, are operational. (See Figure 11.)

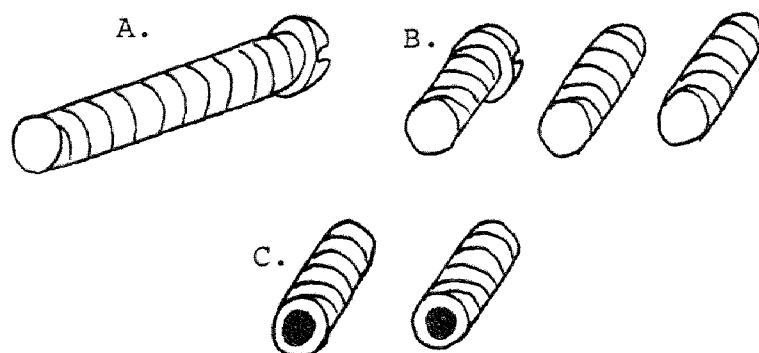


Figure 11. Construction of the guide tubes.

Cap. The cap, when screwed onto the guide tube following the implantation procedure, protects the dura from the outside. It is removed only for ventricular infusions or cleaning of the guide tube. The cap is prepared from a #440 brass bolt and a #440 brass nut. The bolt is inserted into the nut one turn and the two are soldered together. The bolt is cut 3 mm from where it makes contact with the nut. Extra solder and the rough edges of the assembly are filed smooth. (See Figure 12.)

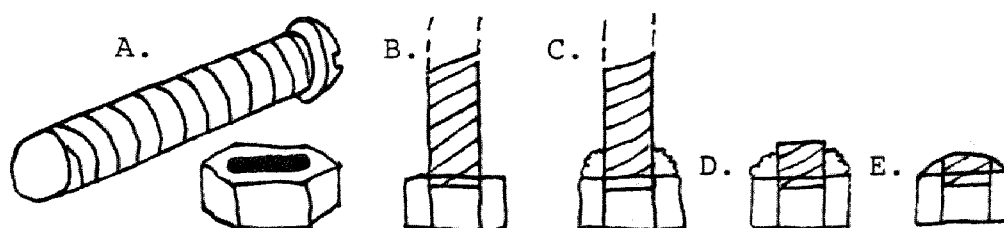


Figure 12. Construction of the guide tube cap.

Infusion Probe. The infusion probe is constructed from a #440 brass bolt, two #440 brass nuts, a 5/8 inch 25 gauge needle, and a durable rubber adhesive.

The brass bolt is drilled out as above to a diameter of 1/16 of an inch and inserted one half of one turn into one of the brass nuts. The two are soldered together and the bolt sawed off as close to the nut as possible. The remainder of the screw and solder protruding from the nut is filed to within 0.5 mm of the surface of the nut. The construction of this prepared nut is

illustrated in Figure 13.

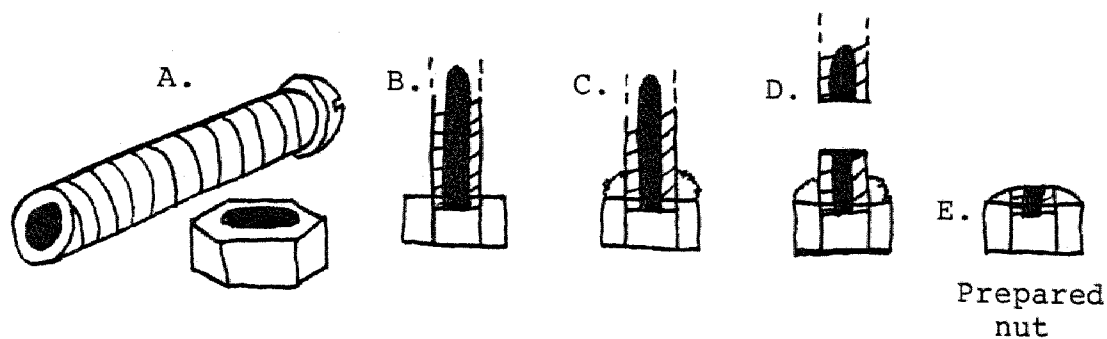


Figure 13. Construction of prepared nut.

The prepared nut and a second nut are arranged on a brass bolt so that they are touching and glued together using the rubber adhesive. When dry, this screw assembly is removed from the bolt, as in Figure 14.

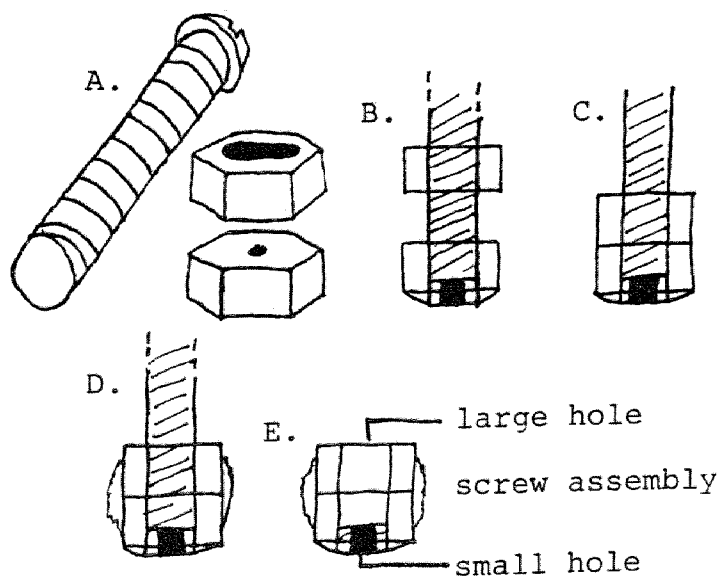


Figure 14. Construction of screw assembly.

The needle and plastic collar of the 25 gauge needle are separated avoiding damage to either piece. A piece of white plastic adhesive on the needle will be used as a stop preventing the needle from moving in the infusion probe assembly. The blunt end of the needle is inserted into the large hole of the screw assembly until the stop makes contact with the piece of the #440 brass bolt in the prepared end of the screw assembly. The blunt end of the needle should pass through the 1/16 inch hole and protrude one to three millimeters past the surface of the screw assembly. The plastic cap is repositioned on the blunt end of the needle and pushed flush with the surface of the screw assembly. The assembly is glued to the cap with the rubber adhesive.

Each infusion probe is matched with a guide tube, labelled, and screwed together until the guide tube cannot be inserted any further into the probe. The end of the needle shaft is filed so that only 3 mm protrude from the base of the guide tube. (See Figure 15.)

Infusion Tubing. Polyethylene tubing with an inner diameter of 0.86 mm and an outer diameter of 1.27 mm is equipped with adaptors at either end to attach to the infusion probe and T-chamber.

One end of the tubing is equipped with a male end which fits into the plastic cap of the infusion probe as seen in Figure 16. The barrel of a one milliliter syringe

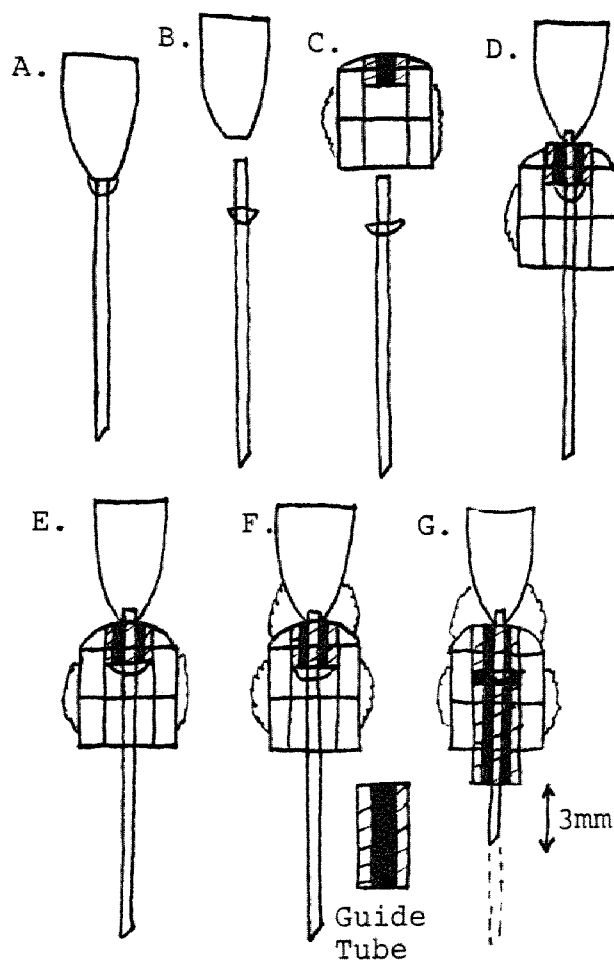


Figure 15. Construction of the infusion probe.

is cut 2 cm from the tip of the needle end. The tubing is inserted through the cut end until the end protrudes past the end of the tube tip 1 cm. Rubber cement is squeezed through the sawed end of the syringe barrel around the tubing until it begins to pass out of the tube tip around the tubing. The cement must pass completely through the tube to the end to insure that no air pockets develop. Once the cement is dry, the extra tubing is cut flush with

the tip of the syringe barrel and the tubing is checked for clear passage.

The other end of the tubing is connected to an 18 gauge needle and cemented with rubber adhesive as seen in Figure 17.

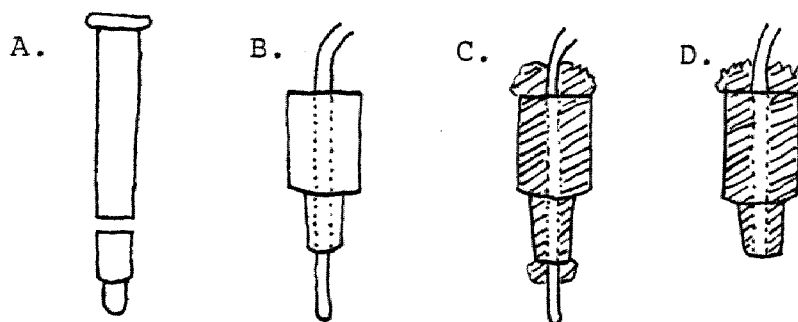


Figure 16. Construction of infusion probe adapter union on infusion tubing.

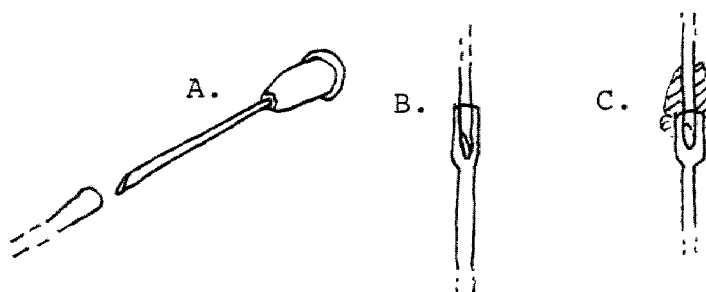


Figure 17. Construction of T-chamber adapter of infusion tubing.

T-chamber. The T-chamber serves as a common pathway for the production and measurement of pressure of the infusion system. It connects the infusion pump, infusion

tubing, and pressure transducer permitting measurement of minute changes in the pressure of the fluid being delivered. It is constructed from two syringes, one 10 ml and one 5 ml, one 18 gauge needle, one 20 gauge needle, and silicone adhesive.

Both syringes are cut 10 mm from the male end of the barrel and a 1/16 inch hole drilled 5 mm from the sawed end in each. With the sawed ends facing each other, the small syringe is inserted into the large syringe and the drill holes are aligned. An 18 gauge needle is inserted through both holes with the tip protruding into the lumen of the 5 ml syringe. Silicone adhesive is squeezed between the two syringe barrels filling the space between them, but not passing into the lumen of the 5 ml syringe. Sealer is also applied around the needle from where it enters the 10 ml syringe barrel to the plastic collar. When the sealer is dry, the 20 gauge needle, which will receive the needle from the microsyringe, is positioned on one of the male ends as seen in Figure 18.

Operation of the Guide-Tube-Infusion System

Filling the System. After the infusion system is assembled as illustrated in Figure 19, the open end of the infusion tubing is placed into a saline reservoir. With both stopcocks open, the control syringe is gently withdrawn drawing saline through the tubing filling the T-chamber and pressure transducer. When they are full and

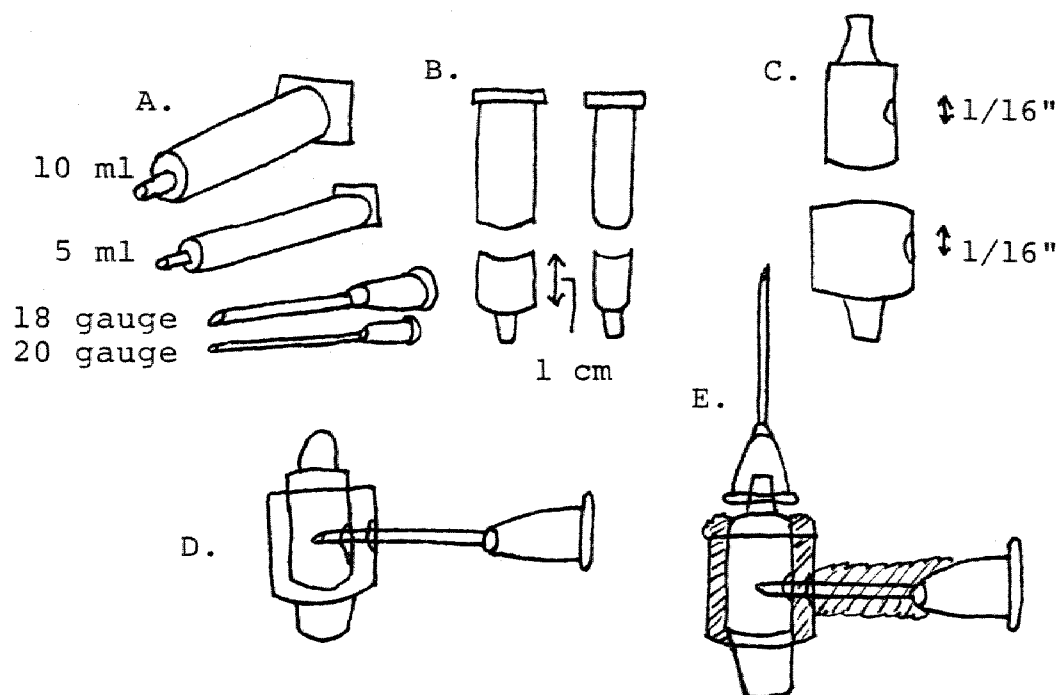


Figure 18. Construction of T-chamber.

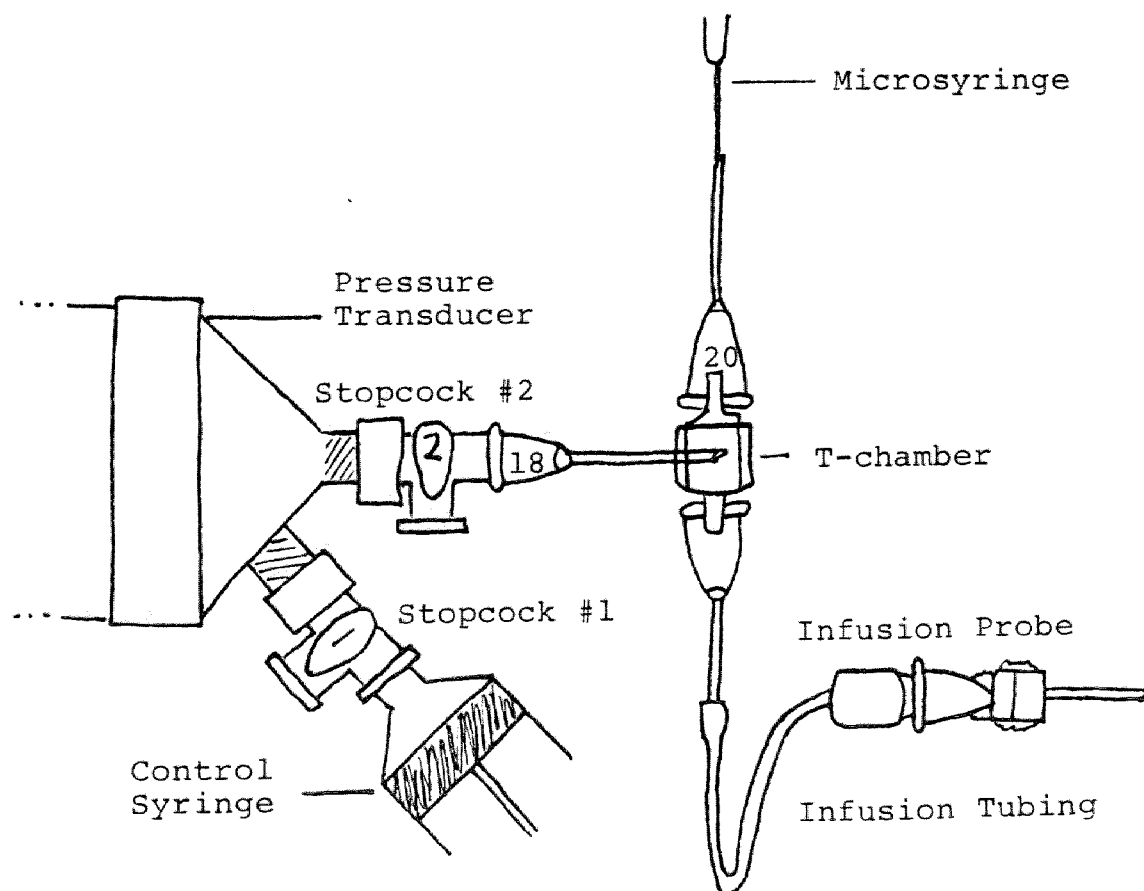


Figure 19. Proper assembly of all infusion system components.

all bubbles are evacuated, stopcock #1 is closed. To fill the microsyringe, the open end of the infusion tubing is capped and the plunger is removed from the microsyringe. Stopcock #1 is reopened, and the control syringe pushed in, driving saline through the 20 gauge needle into the microsyringe. When the syringe is completely filled, stopcock #1 is closed and the microsyringe plunger reinserted. The end of the infusion tubing is uncapped and placed into saline allowing equalization of pressure in the system.

System Sensitivity. The critical feature of the infusion technique is the ability to detect minute pressure changes in the fluid being delivered as the infusion probe tip penetrates the lateral ventricle. The recorder must be sufficiently sensitive to indicate a change in pressure resulting from the delivery of 0.001 mm of fluid. Since values are so close to zero, calibration is not practical.

Checking the System for Leaks. The end of the infusion tubing is capped and, with stopcock #1 closed, a volume of fluid delivered with the microsyringe, causing a measurable increase in the pressure of the system as detected by the strip-chart recorder. Delivery is halted and this pressure is maintained for at least two minutes. The record is examined for a gradual drop in pressure toward zero, characterizing leakage. If the system maintains a steady baseline for a two minute period, any leakage is of a degree that it will not significantly effect the operation

of the system. A noteable drop must be traced and plugged using silicone sealer.

Loading the System. During this process, the sample to be infused is loaded into the infusion system for delivery.

With the system filled and the probe tip of the infusion tubing in the saline reservoir, the microsyringe plunger is depressed expelling into the reservoir a volume equal to the contents of the syringe barrel. The probe tip is removed from the reservoir and placed into a vial containing the substance to be infused. This is drawn into the infusion system by pulling back the plunger of the microsyringe to the desired volume. The probe tip is left in the infusion sample vial until the time of infusion to prevent air bubbles from entering the tubing.

Infusion Delivery. The infusion system is equipped with a syringe pump mechanism in the form of a motor drive attachment to the microsyringe. This device features variable delivery rates and promotes uniform delivery of the infusion sample.

Implantation Procedure. The materials used in the guide tube implantation procedure are listed in Appendix 2.

Male rats weighing 225 to 275 gm are anesthetized using a 40 to 50 mg/kg dosage of Nembutal injected intraperitoneally. The first injection of 40 mg/kg is administered, and if needed, a second injection of 10 mg/kg is

given.

The rat's head is shaved from the base of the skull to the forehead, and placed into a stereotaxic apparatus. A midsagittal incision 2 cm long is made and the skull is exposed. A 1.2 mm diameter hole is drilled 1 mm lateral and 1 mm posterior to the bregma with care taken to avoid damage to the dura. This hole is shaped to permit threading of the guide tube into the skull.

The infusion probe-guide tube set is screwed together, the infusion system is primed, and the combined probe-guide tube assembly is started into the hole. When the threads of the guide tube first firmly bite into the bone, inward progress is stopped, and the infusion pump started at a rate of 6.6 $\mu\text{l}/\text{min}$. Because of the density of the brain tissue in which the probe tip rests, the system is effectively blocked, and as the pump continues to deliver fluid, the pressure of the system increases. When the pressure change causes a 1 cm deflection of the pen on the strip chart recorder, the infusion apparatus is slowly screwed into the skull at a rate of $\frac{1}{4}$ turn per second. Slow inward progress will reduce the chance of overshooting the ventricle. When the tip penetrates the lateral ventricle, the blockage at the end of the guide tube is removed, and the pressure dramatically drops to almost zero. Inward progress is halted at this point. To confirm penetration of the ventricle, the infusion pump speed is doubled for

one minute. A smooth and relatively even pressure line is characteristic of even flow through the ventricular system. With ventricular penetration confirmed, the guide tube is held with a hemostat and the infusion probe unscrewed from the assembly leaving the guide tube imbedded in the skull at this specific depth. This permits rapid and accurate relocation of the lateral ventricle with that same infusion probe.

Starter holes for two anchor screws are drilled in the midsagittal plane 6 mm anterior and posterior to the guide tube. The anterior screw is implanted with half of the shaft length protruding above the skull and the posterior screw with $2/3$ of its length above the bone. Dental resin is applied around the base of the guide tube and anchor screws securing the three as a unit.

Powdered antibiotic is applied and the area sutured bringing the edges of the incision into contact with the resin border. The guide tube is gently irrigated with saline to float out any debris and is capped. The rat is returned to its cage to recover for a two week period during which the light-dark cycle is maintained.

Infusion Procedures

Handling and Maintenance of the Guide Tubes. The infusion procedure entails the insertion of probes into the lateral ventricles of unanesthetized rats. Pappenheimer

showed that these animals will permit this procedure without struggle once accustomed to being handled. It is, therefore, imperative that the rats be accustomed to handling and activity in the area of their guide tube for this technique to be successful.

The guide tubes must be cleaned twice a week to prevent blockage. In this procedure, the rat is removed from its cage and handled for several minutes. The guide tube cap is removed and the guide tube slowly irrigated with warm saline with care taken to avoid puncturing the dura. When the tube is clear, the cap is replaced and the rat returned to its cage. This procedure also aids in familiarizing the animal with handling.

Ventricular Infusion in Rats. The intraventricular infusions are conducted between 1530 and 1700 hours. The infusion system is filled, tested, and loaded with 0.15 ml of the infusate. As in the guide tube cleaning procedure, the rat is removed from its cage and handled briefly, and the guide tube cap is removed. The tube is irrigated with warm saline to insure clear passage of the probe. With the infusion pump delivering at a rate of 6.6 μ l/min, the infusion probe is started into the guide tube. As the probe tip enters brain tissue, signalled by a slow increase in the pressure of the system, inward progress is delayed to accumulate sufficient pressure to deflect the pen 1 cm. When this pressure has been attained, the probe is inserted

by screwing the infusion apparatus onto the guide tube at a rate of $\frac{1}{4}$ turn per second. Infusion pressures are monitored continuously as an indication of probe tip location in the brain. Entry into the ventricle, signalled by a sudden drop in the accumulated pressure, should occur just prior to the point where the probe cannot be turned farther onto the guide tube. Care must be taken not to overturn the assembly as the guide tube may become dislodged from the resin, altering probe tip position.

When entry is confirmed, as described in the implant procedure, the infusion rate is cut to 3.3 $\mu\text{l}/\text{min}$ and the remaining portion of the infusate delivered over the next 30 minute period. During the infusion, the rat is either held or placed in a storage cage and observed. When the process is completed, the probe is removed, and the guide tube is capped. The rat is then returned to its cage and allowed to settle down to its normal daylight state of relative quiescence. Locomotor activity is not recorded until 1800 when the dark phase of the diurnal cycle begins.

Each rat will be allowed to recover fully from the effects of an infusion before being used for another assay. The criterion for complete recovery is the resumption of normal locomotor activity for at least two days and nights.

Confirmation of Guide Tube Placement. A technique has been devised by Pappenheimer to insure proper guide tube placement and to verify that the infusate has been

reaching the entire ventricular system. Each rat will be used for up to five infusions. Two days after the last infusion procedure, each rat will be sacrificed fifteen minutes after being infused with 0.15 M saline and methylene blue. The brain is immediately removed, sectioned to expose the ventricles, and examined to insure that the dye reached all four ventricles.

RESULTS

Factor S Donors

Sleep Deprivation Device. Familiarity of the dogs with this apparatus before the experimental procedures eliminates much initial stress by insuring that the dog has learned to avoid being shocked by standing. Three dogs were placed in the device for a period of six hours, during the light phase of their diurnal cycle, late in the acclimation period. It was found that after being familiarized in this manner, the animals learned, usually within the first two hours, that a shock is avoided by remaining in a standing position, and carefully avoided lying down in subsequent sleep deprivation procedures. The average time that current was delivered was nine minutes of the six hour period. No signs of injury or change in attitude toward the handlers was observed following this process.

This sleep deprivation apparatus was used to deprive several dogs of short periods of sleep. Extended sleep

deprivation was conducted on two dogs, each being deprived of 36 hours of sleep. The average time that current was delivered to these acclimated animals was 15 seconds for the entire procedure.

Because situations can arise which allow the dog to sleep, such as displacement of the shock plates, it was necessary to remain within earshot of the cage buzzer when conducting sleep deprivation procedures. If the animal sleeps, the data is rendered unacceptable. By remaining near, one can rectify problems as they arise.

Exercise Apparatus. The treadmill fulfilled the criteria established for the exercise apparatus to be used in this project. It provided a method to deliver exercise that was uniform in both rate and amount. Because it is used in a controlled environment and manner, the influence of novel stimuli upon the data is greatly reduced. Four dogs were exercised, as described in the "exercise" experimental procedure, for six hours of the twelve hour light phase. Afterwards, the animals appeared fatigued, but were healthy and responded favorably to handling.

Because the treadmill presents an unnatural form of exercise and the dogs may initially become confused and agitated, it is important for the animals to become familiar with the treadmill. This process was carried out in seven sessions spread throughout the acclimation period. In the first session, which usually proved most difficult, the

treadmill was run for several ten second intervals to allow the animal to become familiar with starting and stopping the device. The following six sessions were increased in duration from ten minutes to one hour by ten minute increments. Thirteen dogs were acclimated utilizing this technique. This process satisfactorily prepared them for the experimental procedures.

CSF Withdrawal. Tube curare satisfactorily immobilized the dog for the cisternal puncture. Better control was obtained in cisternal puncture. Better control was obtained when the drug was administered in two doses, the second of which is given only if needed. The tubocurare acted almost immediately once a threshold level in the blood was reached. The amount of time between the onset of paralysis and when the drug affected the respiratory muscles proved adequate in which to perform the cisternal puncture.

Neostigmine Methyl Sulfate adequately reversed the effects of the curare. The wagging of the dog's tail is generally the first indication of the drug's action. Usually within twenty minutes, the animal regains complete motor control. Several dosages, ranging from 0.25 to 1.5 mg/animal, were used. It was found that a 0.5 mg/animal dosage, regardless of the size of the dog, was most effective. With less than this amount, the effects of the neostigmine were often either not pronounced or unacceptably

slow. When dosages greater than 0.5 mg/animal were given, overdoses would sometimes occur resulting in death.

A total of twelve cisternal tap procedures were performed upon eight dogs. Five dogs had one cisternal puncture, two had two punctures, and one had three cisternal punctures. No adverse effects became apparent in either the short or long term with any of these dogs.

The total time from the administration of the first dose of curare to the complete return of motor control to normal with the neostigmine ranged from seven to 55 minutes. The average time was 31 minutes.

Storage of CSF. As with Pappenheimer, the CSF samples were transferred to sterile vials, labelled, and stored at -20°C until the assay procedure. The samples obtained were frozen and thawed several times. No changes in consistency were observed.

Factor S Recipients

Activity Cages. The activity cages described in this paper and those used by Pappenheimer differ significantly only in the manner in which the data is recorded. Pappenheimer used a counter system which triggered each time either of the activity cage photoelectric beams was broken. This counter was photographed at six hour intervals, providing four numerical values per 24 hour period upon which to base locomotor activity.

The system devised in this laboratory, as described in the methods section, is superior to Pappenheimer's in two respects. First, a permanent and complete record of each individual beam break is obtained, allowing more complete analysis of the data if desired. Secondly, because each light beam is connected separately, the record gives a gross indication of the rat's position in the cage.

The disadvantage of the strip chart method is the amount of time and effort needed to analyze the records. Each pen deflection, indicating a break in its respective light beam, must be counted per unit time. Pappenheimer's system circumvents this by presenting the data in a very condensed form.

The data required in this project are not of a nature which greatly benefit from the additional information provided by the strip chart method. Therefore, either method will adequately fulfill the requirements of this project.

Guide Tube and Infusion Probes. The guide tube-infusion probe system described in this paper provides two advantages over Pappenheimer's system. First, the design and construction permits both the implantation of the guide tube and the measurement of the ventricular depth in one step instead of two. Second, this design allows uniform construction of the guide tubes and infusion probes rather than custom preparation for each rat.

This apparatus satisfactorily permitted repeated penetration and infusion into the lateral ventricles of rats.

Implant Procedures. This procedure permits implantation of the guide tube in an average time of 30 minutes. It proved to be consistently simple and successful. All of the seven rats that were implanted using this procedure survived.

If the guide tube assembly is not securely anchored, it may pull out of the skull with the first or second infusion. Several modifications of anchoring were explored before the two screw method similar to Pappenheimer's was devised. This technique satisfactorily secures the guide tube for the duration of the project.

Infusion Procedures. The infusion procedures provide a relatively simple means of rapid relocation and infusion of the lateral ventricle of rats.

After infusing three rats in this manner, it was found that the pressure drop, described in the methods, resulting from the penetration of the probe tip into the lateral ventricle was obvious and repeatable. The method used to verify ventricular penetration, in which the normal infusion rate is doubled, also proved satisfactory.

Methylene Blue Verification. This method, as described by Pappenheimer, demonstrated flow of the infusate throughout the entire ventricular system. In the one rat

that was infused in this manner, the methylene blue clearly stained all four ventricles.

SUMMARY

This paper provides a means of evaluating the effects of sleep deprivation and exercise upon the production of sleep promoting substance in the CSF of dogs. The relative amount and effectiveness of this substance is ultimately evaluated, via the Pieron Phenomenon, by comparative activity studies in recipient rats. The project incorporates several published techniques, some modifications of those, and several successfully developed new techniques. When appropriately combined, they can be incorporated into subsequent experiments measuring the effects of a variety of parameters on the production of sleep promoting substance in the CSF.

LITERATURE CITED

- Baekeland, R., and R. Lasky. 1966. Exercise and sleep patterns in college athletes. *Perceptual and Motor Skills* 23:1203-1207.
- Berger, R. J., and I. Oswald. 1962. Effects of sleep deprivation on behavior, subsequent sleep, and dreaming. *J. Ment. Sci.* 108:457-465.
- Fisher, C. 1965. Psychoanalytic implications of recent research on sleep and dreaming. *J. Amer. Psychoanal. Assn.* 13:197-270.
- Kales, A., T. Wilson, M. J. Paulson, J. D. Kales, and R. D. Walter. 1966. Relationships between age and sleep patterns. Paper presented at meeting of Assoc. for the Psychophysiological Study of Sleep, Gainesville, Fla.
- Oswald, I. 1962. *Sleeping and Waking*. Elsevier Pub. Co., Amsterdam, New York. 232p.
- Pappenheimer, J. R. 1976. The sleep factor. *Sci. Am.* 235(2):24-29.
- Roffwarg, H., W. C. Dement, and C. Fisher. 1964. Preliminary observation of the sleep-dream pattern in neonates, infants, children, and adults. In E. Harms (ed.), *Problems of Sleep and Dreams in Children*. Vol. II. Bergaman Pub. Co., New York. n.p.
- Roffwarg, H., W. C. Dement, and C. Fisher. 1966. Ontogenetic development of human sleep-dream cycle. *Science*. 152:604-619.
- Williams, R. L., H. W. Agnew, Jr., and W. B. Webb. 1966. Relationships between age and sleep patterns. Paper presented at meeting of Assoc. for the Psychophysiological Study of Sleep, Gainesville, Fla.

APPENDIX 1

CSF WITHDRAWAL MATERIALS

Tubocurare - 0.1 to 0.3 mg/kg of body weight

Neostigmine Methyl Sulfate - 0.5 ml of 0.1%

solution/animal

3 x 20 gauge needles

sterile storage vial

1 x 1 ml syringe

2 x 5 ml syringe

1 x 10 ml syringe

APPENDIX 2

GUIDE TUBE IMPLANTATION MATERIALS

rat - male, 225-275 gm

Nembutal - 5% solution

1 x 1 ml syringe and 25 gauge 0.5 inch needle

dental drill and bits

surgical tools

2 x 0.25 inch brass sheet metal screws

stereotaxic apparatus

metric ruler and pen

infusion tubing, microsyringe, pump, T-chamber

strip chart recorder, pressure transducer

2 x 3 way stopcocks

powdered antibiotic

dental resin